

ENHANCEMENT OF *PASTEURELLA HAEMOLYTICA* Δ 1
LEUKOTOXIN ACTIVITY BY BOVINE
SERUM ALBUMIN

BY

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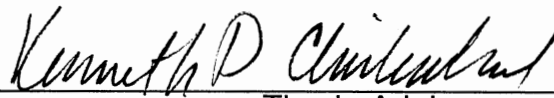
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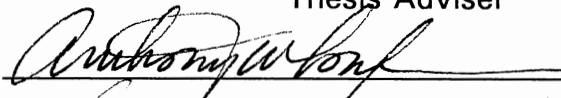
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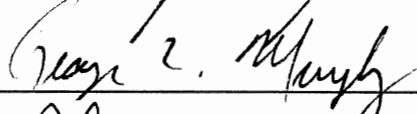
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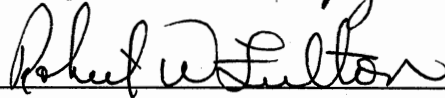
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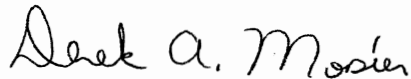


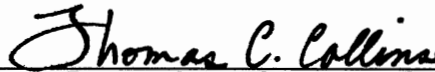
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ABSTRACT

Growth of *Pasteurella haemolytica* A1 in media containing fetal bovine serum has been observed to enhance leukotoxin (LKT) activity, but the mechanism of this increase is not understood. We found that bovine serum albumin in the absence of other serum components also enhances LKT activity. During the logarithmic growth phase, LKT activity was $30,700 \pm 12,900$ Toxic Units (TU)/ml for *P. haemolytica* grown in RPMI medium containing 0.5% bovine serum albumin (BSA) (BSA-LKT), compared to 120 ± 40 TU/ml in medium containing RPMI alone (RPMI-LKT). In other experiments, addition of 0.5% BSA to RPMI-LKT culture filtrate (RPMI-LKT/BSA) resulted in LKT activity intermediate ($13,000 \pm 1,600$ TU/ml) between that of BSA-LKT ($55,600 \pm 11,500$ TU/ml) and RPMI-LKT ($2,200 \pm 900$ TU/ml). The activity of RPMI-LKT, BSA-LKT and RPMI-LKT/BSA decreased when the toxin preparations were incubated at room temperature (25°C) for 2 hours; however, the decrease in RPMI-LKT activity was more pronounced. Concentrated toxin from logarithmic growth phase RPMI-LKT culture supernatants contained a single LKT activity peak (Peak I) on Sephacryl HR-400 which had a K_{av} of 0.01 and estimated molecular weight $> 669,000$. In contrast, gel filtration of concentrated toxin from logarithmic growth phase BSA-LKT culture supernatants had

three activity peaks with K_{av} values of 0.04 (Peak I), 0.66 (Peak II), and 0.87 (Peak III). Gel filtration of RPMI-LKT/BSA also consisted of Peaks I, II, and III. Peaks I, II, and III contain a prominent 97000 band on SDS-PAGE which was identified as LKT by Western blot analysis with an monoclonal antibody (MAB) to LKT. The identity of Peaks I, II, and III were further confirmed as LKT by target cell specificity (lysed bovine lymphoma cells and not equine leukocytes) and neutralization with a MAB to *P. haemolytica* LKT. It is concluded that LKT produced in RPMI medium alone is a large aggregate which undergoes conformational changes in the presence of BSA resulting in at least two additional LKT forms which may be partially disaggregated forms of the large aggregate LKT produced in RPMI.

INTRODUCTION

Pasteurella haemolytica biotype A, serotype I, is the most common strain of bacteria isolated from cattle with bovine pneumonic pasteurellosis (shipping fever pneumonia). This bacterium produces several structural and nonstructural components important for production of disease including endotoxin, polysaccharide capsule, outer membrane proteins and an exotoxin (leukotoxin) (8).

Leukotoxin, a heat-labile immunogenic exotoxin produced by *Pasteurella haemolytica* A1 during its logarithmic growth phase (8), is toxic to ruminant leukocytes and may act to impair pulmonary defenses or incite undesirable inflammatory reactions (8). Leukotoxin is a member of the repeats in toxin (RTX) family of gram negative bacterial cytolysins (23). The nucleic acid sequences of the polycistronic gene coding for activation, structural, and transport proteins of *P. haemolytica* LKT and the prototype RTX toxin *E. coli* α -hemolysin are similar, sharing 66, 62, 90.5 and 75.6% sequence homology, respectively (11,17,22). Members of the RTX family injure target cells by the formation of transmembrane pores resulting in osmotic lysis (4-7). In addition, at sublytic concentrations, LKT is a potent neutrophil modulating agent which can stimulate the release of neutrophil oxidative intermediates, preferential release of secondary granule constituents, and induce cytoskeletal alterations in bovine neutrophils (9). LKT is distinguished from other RTX cytolysins by its narrow target cell specificity: ruminant leukocytes and platelets (4-7,15,20). Although the calculated molecular weight for LKT, from its gene sequence is 105,000, the primary

band identified in culture supernatants of *P. haemolytica* examined by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) is a 97,000 protein (3). The structure of the native LKT is uncertain, but it appears to be a multimer of this 97,000 protein (3). Molecular weight estimates of the native LKT have ranged from 150,000 to >300,000 (2,13,17).

Growth of *P. haemolytica* A1 in medium supplemented with fetal bovine serum results in enhanced toxinogenesis (12). Based on this enhancement of LKT activity by inclusion of nonheme or heme iron binding proteins, iron availability was judged important for toxinogenesis (12). We also observed that addition of bovine serum albumin (BSA) in the absence of other serum components to *P. haemolytica* culture medium resulted in enhanced LKT activity (24). To examine the mechanism for BSA enhancement of toxin activity from *P. haemolytica*, the molecular weight of LKT from *P. haemolytica* grown in media with and without BSA were compared. In this report, we also describe a partial purification procedure with ammonium sulfate concentration of the LKT followed by gel filtration chromatography, which allowed us to obtain biologically active, partially purified toxin.

MATERIALS AND METHODS

Preparation of *Pasteurella haemolytica* A1 culture supernate leukotoxin. Brain heart infusion (BHI) agar (Difco Laboratories, Detroit, MI) plates supplemented with 5% bovine blood were inoculated with a stock culture of *P. haemolytica* A1 (stored in 10% glycerol + BHI broth at -70°C) and grown overnight (18 hours) at 37°C, in 5% CO₂ (12). BHI broth (100 ml/500 ml Erlenmeyer flask) was inoculated from the blood agar cultures to an OD₆₅₀ of 0.20-0.35, and the bacteria were grown at 37°C with 80 oscillations/min until the OD₆₅₀ reached 0.80-1.0. Bacteria were collected by centrifugation (Sorvall, GS3 rotor, 8000 rpm) for 20 minutes and resuspended in 10 ml of either RPMI-1640 medium (RPMI), pH 7.2 or RPMI, pH 7.2, containing 0.5% BSA (RPMI-BSA), (Fraction V, bovine serum albumin, Sigma Chemical Company, #A-6003). These cultures were used to inoculate Erlenmeyer flasks containing either RPMI or RPMI-BSA to an OD₆₅₀ of 0.200-0.350. RPMI and RPMI-BSA were buffered with 2.0 g/L of sodium bicarbonate (Sigma Chemical Company), and the pH adjusted to 7.0 prior to filter sterilization (Nalgene cellulose acetate, 0.2 µm filter); final pH after filtration was 7.2. The pH of the RPMI medium was checked before and after addition of BSA, and did not change upon addition of BSA. The total volume inoculated was always 20% of the flask capacity, and flask number and size were adjusted depending on the quantity of LKT needed; i.e.: (a) for gel filtration experiments, 400 ml medium in 2L flasks was inoculated with *P. haemolytica*, and (b) for timed experiments, 5 replicates of each type media were inoculated, 100 ml medium in 500ml flasks. Cultures were

incubated in an environmental orbital shaker incubator at 37°C, 80 oscillations/min, and grown to an OD₆₅₀ of 0.80-1.01 (or for a specific period of time). For timed experiments, the supernatant was collected by filtration with a 0.2µm cellulose acetate (Nalgene) filter. For gel filtration experiments, the supernatant was collected by centrifugation (Sorvall, GS3 rotor, 8000 rpm), filtered with a 0.2µm cellulose acetate filter and precipitated with ammonium sulfate at 50% saturation (313 g/L) (J.T. Baker Chemical Company, Phillipsburg, NJ). The precipitate was collected by centrifugation (Sorvall, GS3 rotor, 11,500 rpm) for 40 minutes, resuspended in 4.0 ml of column buffer (10 mM Tris, 50 mM NaCl, pH 7.3), and filtered with a 0.2 µm cellulose acetate filter. RPMI-LKT for gel filtration was also concentrated approximately 40X with a stircell apparatus (Amicon), with a Diaflo YM 10 membrane (molecular weight cutoff of 10,000), (Amicon, W.R. Grace & Co., Danvers, MA).

Leukotoxin Assay. Bovine lymphoma cells (BL-3 cells, G. Theilen, University of California, Davis), used as target cells, were exposed to LKT in non-diluted or diluted column fractions or culture filtrate by one of two assay formats: (a) in microfuge tubes or 96 well round bottom microtitre plates (total assay volume 250 µl), or (b) 96 well round bottom microtitre plates (total assay volume 200 µl), and incubated for 2 hours at 37°C in a culture incubator. BL-3 cells split 1:1 in fresh media 1 day prior to use in assays, were resuspended in RPMI (pH 7.2), and cell concentration was 2×10^6 BL-3 cells/ml. At the end of the toxin incubation period, test samples were then centrifuged (microfuge tubes: Surespin, Helena Laboratories, 10,000 rpm, 2 minutes; microtitre plates: Sorvall, H1000B rotor, 1000 rpm, 10 minutes), and the supernatant assayed for the cytoplasmic enzyme, lactate dehydrogenase (LDH) by one of three methods: (a) a

chemistry analyzer using a spectrophotometric assay at 340 nm (Centrifuchem (Dupont), with a commercial LDH assay kit (LDL-50 1X concentration; Sigma Chemical Company), (b) a non-thermally controlled colorimetric assay at 490 nm (Vmax, Molecular Devices, 2 minute kinetic assay), using 2-[4-iodophenyl]-3-[4-nitrophenyl]-5 phenyltetrazolium chloride (INT, Sigma Chemical Company #I-8377) 16.7 mg and phenazine methosulfate (PMS, Sigma Chemical Company P-9625) 4.3 mg dissolved in 50 ml of LDL-50 1X concentration (Sigma Chemical Company) (15), or (c) a thermometric spectrophotometric kinetic assay at 340 nm (Thermomax, Molecular Devices, 2 minute kinetic run) (LDL-50 2X concentration, Sigma Chemical Company). BL-3 cells were incubated with 1% Triton (Triton-X, EM Industries) to release 100% of cellular LDH for positive controls. Spontaneous LDH release was determined from BL-3 cells incubated with RPMI. Raw data was reported as mOD/min for the colorimetric and thermometric spectrophotometric kinetic assays, and as IU/L for the spectrophotometric assay using the chemistry analyzer (gel filtration elution profiles). Raw data was converted to Toxic Units (TU) in experiments where quantitation of the LKT was necessary (timed experiments). To determine TU, serial dilutions of the toxin were incubated with the BL-3 cells, the LDH mOD/minute plotted against the reciprocal dilution of the filtrate sample, and the number of TU determined by probit analysis (21). One TU was defined as the amount of LKT required to release 50% of the LDH from 2×10^6 BL-3 cells/ml.

***P. haemolytica* growth and LKT activity.** To examine the effect of BSA on *P. haemolytica* growth and LKT production, five cultures of *P. haemolytica* grown in RPMI medium were compared to five cultures grown in RPMI-BSA medium. Culture growth was assessed by measuring culture turbidity (OD_{650}) at 0, 2.5, 6, 12, and 24 hours of incubation. Aliquots of

filtered RPMI-LKT and BSA-LKT culture supernatant were assessed for cytotoxicity against BL-3 cells by release of LDH. Data were analyzed with the student's t-test (22).

Effect of BSA on RPMI-LKT. To examine the effect of addition of BSA on RPMI-LKT, RPMI-LKT and BSA-LKT were prepared as described from 2.5 hour bacterial cultures. The culture supernatants were filtered, and the RPMI-LKT divided into two equal volumes in 100 ml sterile Pyrex bottles. An aliquot of RPMI-LKT from one of the bottles was removed, and an equal volume of filter sterilized, concentrated (10%) BSA in RPMI was added to bring the RPMI-LKT culture filtrate up to 0.5% BSA (RPMI-LKT/BSA). An equal volume of the BSA-LKT was placed into a sterile 100 ml Pyrex bottle, and a volume equal to that removed from the RPMI-LKT was removed from the BSA-LKT and the second RPMI-LKT preparations and replaced with the same volume of sterile RPMI medium. The BSA-LKT, RPMI-LKT and RPMI-LKT/BSA filtrates were incubated at 25°C in an environmental shaker incubator at 100 oscillations per minute, and triplicate samples from each preparation were assayed for LKT activity after 0, 15, 30, 60, and 120 minutes of incubation. Time 0 was defined as the time of addition of the BSA to the RPMI LKT. Data were analyzed using ANOVA and LSD (22). The above experiment was repeated using low endotoxin BSA (fraction V powder Albumin, Sigma Chemical Company, #A-9543), which contains less than 0.1 ng/mg of protein of detectable endotoxin.

Gel-filtration chromatography. Ammonium sulfate-precipitated and concentrated RPMI-LKT, BSA-LKT or RPMI-LKT/BSA or stircell concentrated RPMI-LKT in a volume of 2.5 ml was loaded onto a Pharmacia K26/70 column packed with Sephacryl HR-400 (Pharmacia). The total bed volume of the column was 336.0 ml. Fractions were collected into tared test tubes

every 15 minutes with a Pharmacia Helirac, and the mean fraction volume calculated for each run. Fractions were eluted with column buffer (10 mM Tris, 50 mM NaCl, pH 7.3) at a flow rate of 20 ml/hour. The column was calibrated with Tobacco mosaic virus (molecular weight 45,000,000) or heat killed *Serratia marscesens* (void volume), thyroglobulin (669,000), ferritin (440,000), catalase (232,000), bovine serum albumin (67,000), carbonic anhydrase (29,000), and ribonuclease A (13,500). The presence of LKT in column fractions was detected by immunoblotting and assaying for LKT activity. Protein was detected by measuring absorbance of column fractions at optical density (OD) 280. Protein content of column fractions was measured with the BCA protein assay using a microtitre plate technique (Pierce Chemical Co.), at OD 540 nm (VMax, Molecular Devices).

Fractions from Peaks I, II and III from BSA-LKT were pooled and concentrated by precipitation with 5% trichloroacetic acid (TCA, Sigma Chemical Company, #T-6399) (final volume/volume) at 4°C for 30 minutes (9). The precipitate was collected by centrifugation (Sorvall, H1000B rotor, 3600 rpm, 10 minutes), resuspended in electrophoresis sample buffer, and the pH of the samples adjusted by addition of 1% NaOH until the indicator dye (bromphenol blue) in the sample buffer turned from yellow to blue (approximate pH 7.0-7.2 determined with pH paper).

Silver stained SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and western blotting. Column fractions or TCA precipitated samples containing LKT activity were diluted 1:2 in sample buffer (electrophoresis sample buffer: 25 ml Tris, pH 6.8, 2 g SDS, 10 ml glycerol, 5 ml 2-mercaptoethanol, and 100 ul of 1% bromophenol blue), denatured by boiling for 1.5 minutes, and subjected to SDS-PAGE, using a 4% stacking gel and a 10% resolving gel (18). The resulting bands were demonstrated by a silver

stain method (25). Duplicate gels were transferred to nitrocellulose membranes and LKT detected with an anti-LKT monoclonal antibody (MAB) against the 97,000 LKT band (MAB601 and/or MAB605b) obtained from Drs. M.J. Gentry and S. Shrikumaran, University of Nebraska) (13). Bands were identified as previously described (18). The MAB was diluted 1:500, the secondary antibody (biotinylated anti-mouse whole molecule IgG, goat derived (Amersham International, RPN 1177) diluted 1:300, and streptavidin-biotinylated horseradish peroxidase complex (Amersham International, RPN 1051) dilution (to detect the reaction) diluted 1:400, with 1% gelatin in tween-phosphate buffered saline, pH 7.5. Bands were demonstrated with horseradish peroxidase color developer (Sigma Chemical Company) (18).

Target cell specificity assay. Fractions from Peaks I, II, and III from BSA-LKT and RPMI-LKT/BSA were assayed for toxicity against equine peripheral blood leukocytes (equine WBC), using BL-3 cells as positive controls. Equine WBC were isolated as previously described (5). Equine leukocytes suspended in RPMI (2×10^6 cells/well) were exposed to LKT from Peaks I, II, and III in 200 μ l final volume for 2 hours at 37°C, and the supernatant assayed for LDH content as described above. Positive controls were the BL-3 cells + CF, BL-3 cells + 1% Triton, and equine WBC + 1% Triton. Negative controls were BL-3 cells + RPMI, and equine WBC + RPMI.

MAB neutralization of LKT activity. The susceptibility of LKT from Peaks I, II, and III from BSA-LKT and RPMI-LKT/BSA to neutralization by an anti-LKT MAB (MAB601) was tested by exposing BL-3 cells (4×10^5 cells/well) to LKT column fractions preincubated with or without anti-LKT MAB. The MAB was decomplexed by heating to 56°C for 1 hour in a water bath. Decomplexed MAB + CF (CF were diluted 1:2, and the MAB was

diluted 1:500) were incubated at 4°C for 30 minutes; BL-3 cells were then added to each well, incubated at 37°C for 2 hours, centrifuged as described, and the supernatant assayed for LDH content. Controls were MAB + BL-3 cells, Triton 1% + BL-3 cells, and BL-3 cells + RPMI.

RESULTS

Enhanced leukotoxin activity in RPMI growth medium supplemented with bovine serum albumin. Logarithmic phase *P. haemolytica* cultures (0 to <6 hours) grown in RPMI medium supplemented with 0.5% BSA, contained $30,700 \pm 12,900$ TU/ml (mean \pm standard deviation) of LKT activity, which was significantly greater ($P < 0.05$) than the 120 ± 40 TU/ml of LKT activity contained in cultures grown without BSA. In contrast, LKT activity (measured in TU) in stationary phase cultures ($t \geq 6$ hours) was not significantly different between the two media ($P > 0.05$) (Figure 1). *P. haemolytica* growth was quantitatively better in RPMI-BSA medium during the logarithmic growth period, compared to growth in RPMI ($P < 0.05$); however, qualitatively the growth curves of *P. haemolytica* grown in RPMI and RPMI-BSA were similar (Figure 1). During stationary growth (6-24 hours), the difference in the growth curve was no longer significant. Growth of *P. haemolytica* in media containing BSA significantly enhances LKT activity.

In another experiment, addition of 0.5% BSA directly to logarithmic growth phase culture filtrates from RPMI media (RPMI-LKT/BSA) resulted in a significant increase ($P < 0.05$) in LKT activity (Figure 2). At time 0, RPMI-LKT/BSA contained 6X more LKT activity than RPMI-LKT, whereas BSA-LKT contained 4-5X more activity than RPMI-LKT/BSA, and 25X more LKT activity than RPMI-LKT; the difference in LKT activity was significantly different between all three ($P < 0.05$). Activity of BSA-LKT, RPMI-LKT and RPMI-LKT/BSA incubated at 25°C was significantly reduced by 120

Figure 1. Enhanced LKT activity in cultures grown in medium containing BSA. (A) During logarithmic growth ($0 < t < 6$ hours), culture supernate from *P. haemolytica* grown in RPMI medium without BSA had 120 ± 40 TU/ml, whereas (B) supernate from cultures grown in RPMI medium containing 0.5% BSA had $30,700 \pm 13,000$ TU/ml. (Open diamonds = bacterial growth, Solid diamonds = LKT activity)

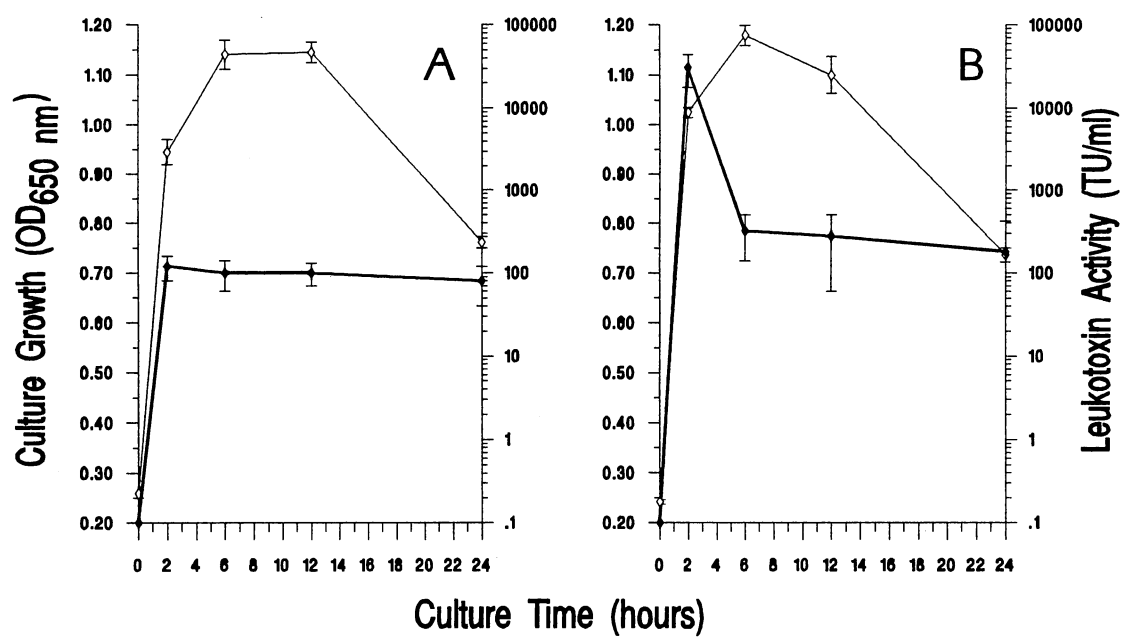
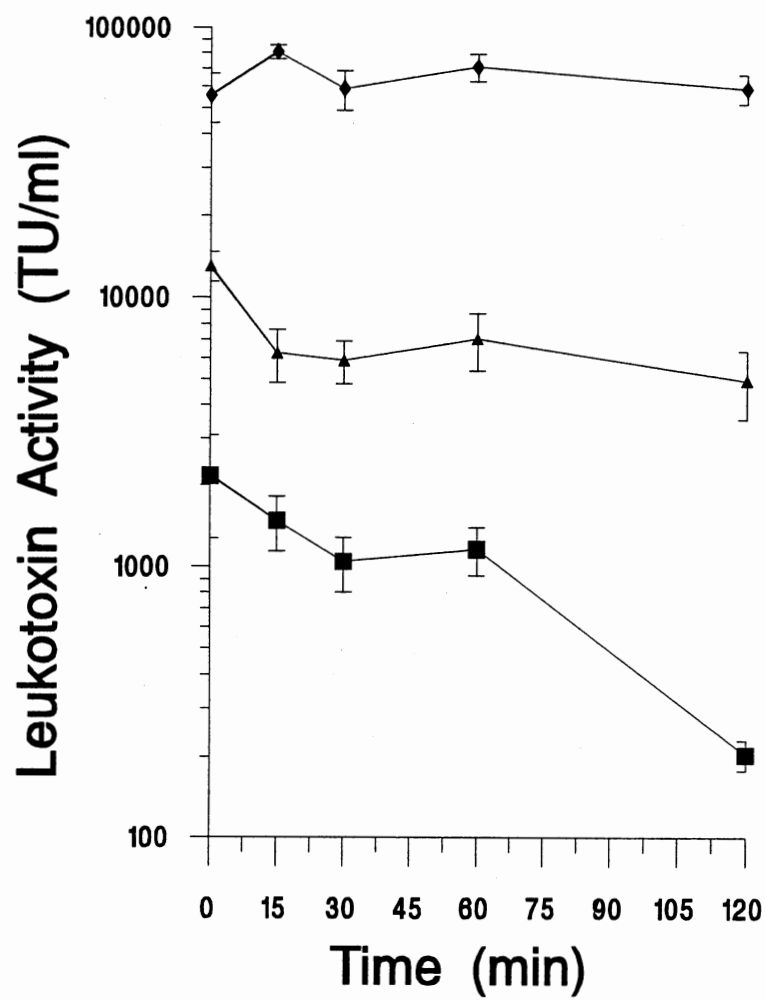


Figure 2. Enhanced LKT activity by addition of BSA to RPMI-LKT culture supernate. At $t=0$, RPMI-LKT/BSA had $13,000 \pm 1,600$ TU/ml of LKT activity, compared to BSA-LKT which had $55,600 \pm 11,500$ TU/ml and RPMI-LKT which had $2,200 \pm 900$ TU/ml. At $t=120$ minutes, activity of all three had reduced, however, the reduction in activity was more pronounced for RPMI-LKT. (Diamonds = BSA-LKT; Triangles = RPMI-LKT/BSA; Squares = RPMI-LKT)



minutes, compared to initial activity at time 0 ($P < 0.05$), but the reduction in LKT activity was most apparent for the RPMI-LKT. Activity of BSA-LKT, RPMI-LKT and RPMI-LKT/BSA between 0 and 60 minutes was not statistically reduced (Figure 2). To make sure that endotoxin levels associated with the BSA preparation were not responsible for the apparent increase in LKT activity, the experiment was repeated using low endotoxin BSA, and the same results were obtained with BSA-LKT, RPMI-LKT and RPMI-LKT/BSA for each time point (data not shown). Therefore, it appears that BSA can act directly on LKT to enhance activity.

BSA enhanced LKT activity is associated with generation of multiple molecular forms. Gel filtration of ammonium sulfate concentrated RPMI-LKT obtained at 2.5 and 6 hours of incubation (Figures 3a & 3c) or stircell concentrated RPMI-LKT obtained at 2.5 hours of incubation (Table 1) resulted in the detection of a single activity peak, which corresponded to a molecular weight $> 669,000$, whereas gel filtration of BSA-LKT obtained at both 2.5 and 6 hours of incubation resulted in recovery of three activity peaks (Figures 3b & 3d). The percentage of LKT activity from BSA-LKT shifted from the higher molecular weight peak to apparent lower molecular weight peaks with increased time of incubation of the *P. haemolytica* cultures (Table 1). Addition of 0.5% BSA directly to culture filtrate from RPMI-LKT (RPMI-LKT/BSA) resulted in an elution profile similar to BSA-LKT (Figure 4, Table 1). It appears that RPMI-LKT exists as a single large molecular weight form and that incubation of *P. haemolytica* in media containing BSA or addition of BSA directly to RPMI-LKT results in LKT existing as multiple forms.

Identification of LKT subunit in various molecular forms of LKT:
Fractions from Peak I from RPMI-LKT and Peaks I-III from BSA-LKT contained

Figure 3. RPMI-LKT and BSA-LKT Elution Profiles derived by gel filtration (Sephacryl-HR 400). RPMI-LKT at 2.5 (A) and 6 hours (C) of culture incubation had a single, low K_{av} peak, corresponding to a molecular weight $>669,000$. BSA-LKT at 2.5 (B) and 6 hours (D) of culture incubation had three peaks, with K_{av} values corresponding to molecular weights of $>669,000$ for Peak I, $>65,000$ for Peak II, and $<29,000$ for Peak III. (Solid line = protein concentration (OD_{280nm}). Triangles = Leukotoxin activity.

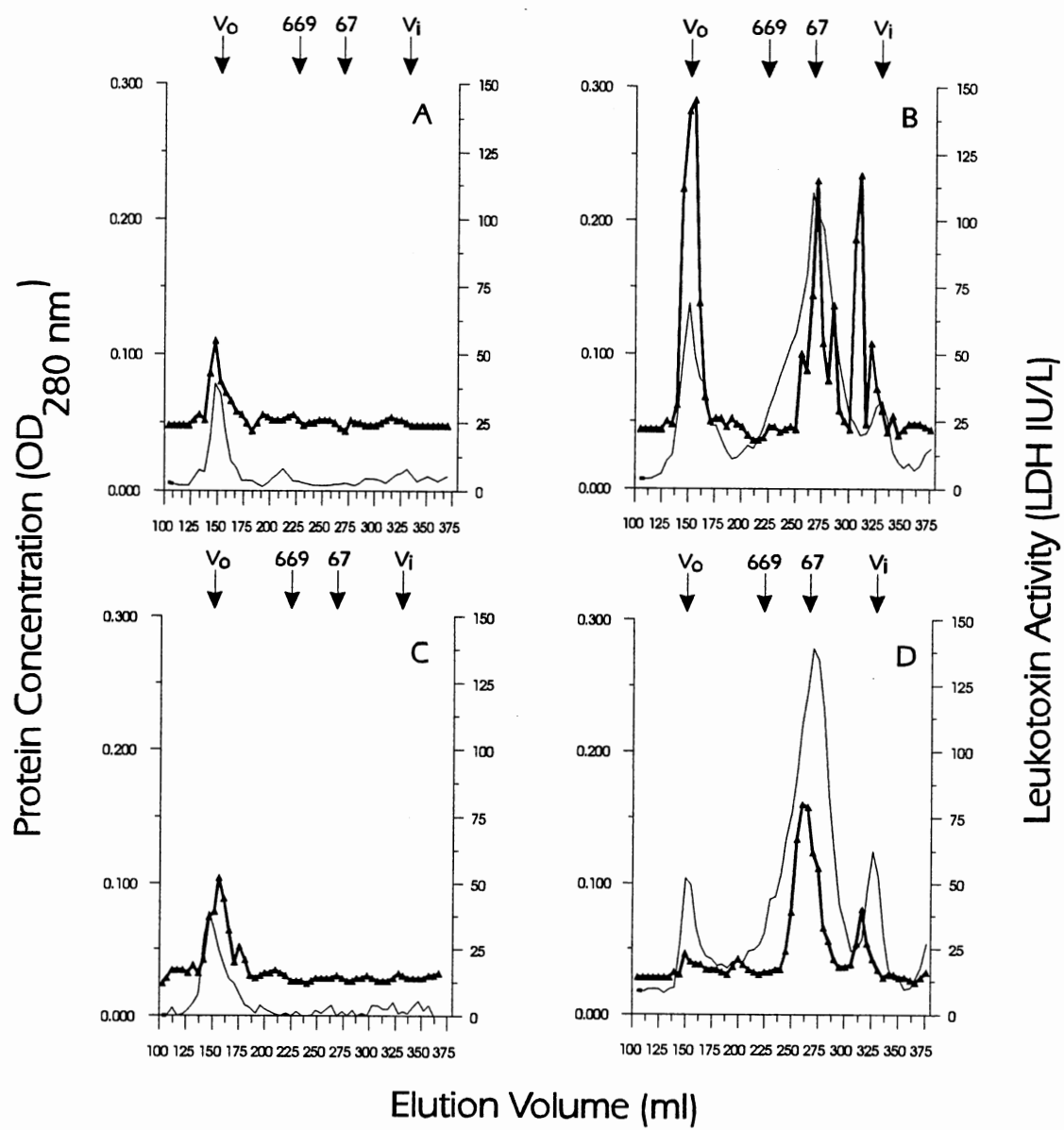


TABLE 1: Summary of Kav Values^a and Percent LKT Activity^b for Fractionated RPMI-LKT, BSA-LKT and RPMI-LKT/BSA on Sephacryl HR-400 Gel Filtration Column

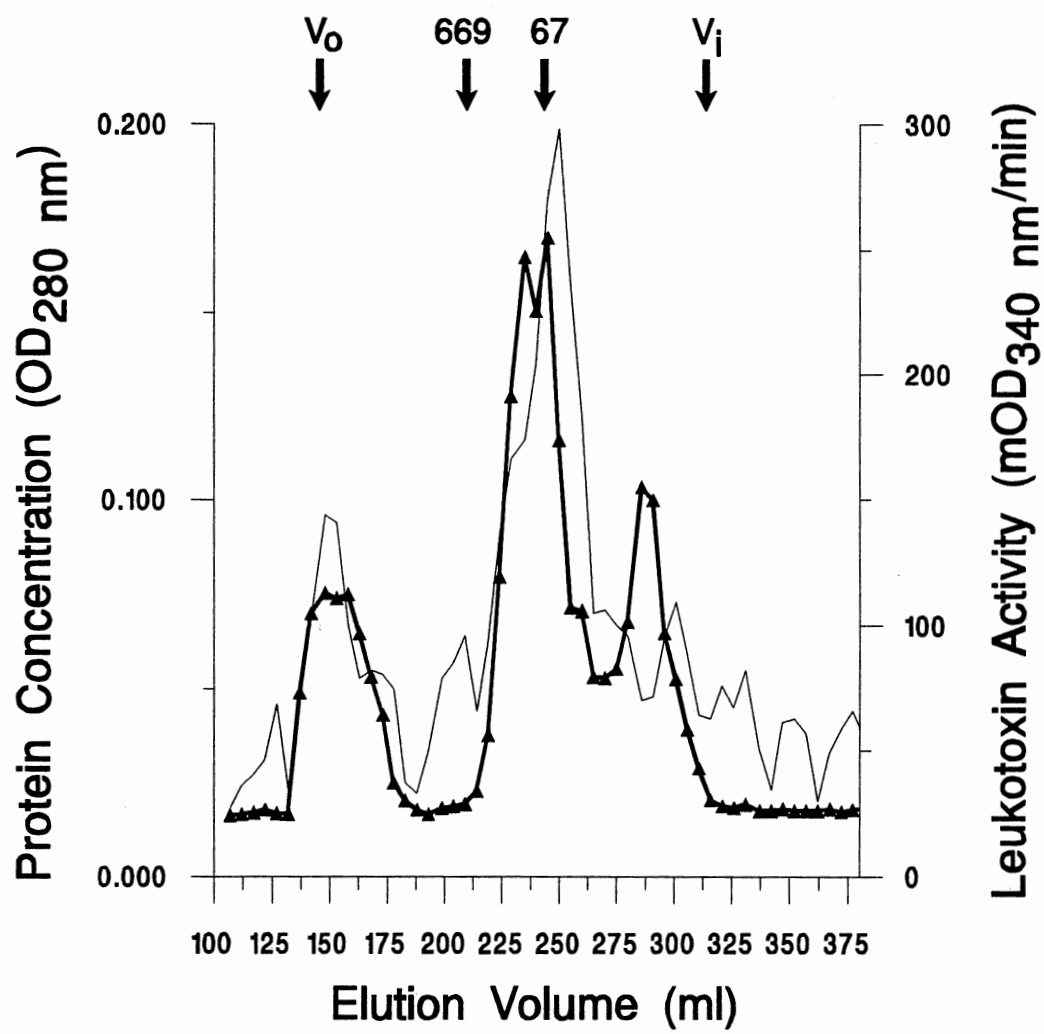
	PEAK I	PEAK II	PEAK III
	K _{av} (% activity)	K _{av} (% activity)	K _{av} (% activity)
RPMI-LKT (AS Conc)			
2.5 hr	0.01 (100%)	----	----
6.0 hr	0.06 (100%)	----	----
RPMI-LKT (Stircell Conc)			
2.5 hr	0.04 (100%)	----	----
BSA-LKT			
2.5 hr	0.04 (40%)	0.66 (35%)	0.87 (25%)
6.0 hr	0.03 (5%)	0.53 (75%)	0.82 (20%)
RPMI-LKT/BSA			
2.5 hr	0.02 (25%)	0.50 (50%)	0.82 (25%)

^aK_{av} represents the fraction of the stationary gel volume which is available for diffusion of a given solute species. $K_{av} = \frac{V_e - V_o}{V_t - V_o}$

where V_e represents the void volume of the column, V_t represents the total volume of the packed bed, and V_o is the elution volume of a particular solute (1), in this case, RPMI-LKT, BSA-LKT or RPMI-LKT/BSA.

^bPercent LKT Activity was determined by calculating the area under the curve for each peak.

Figure 4. The RPMI-LKT/BSA elution profile had three activity peaks, with K_{av} values corresponding to $> 669,000$ for Peak I, $> 65,000$ for Peak II, and $< 29,000$ for Peak III. Solid line = protein concentration (OD_{280nm}). Triangles = LKT activity.



multiple protein bands on SDS-PAGE (Figures 5a & 6a). A 97,000 band was the prominent band for peak I regardless of the source. This 97,000 protein was identified as LKT on western blots using the anti-LKT MAB (Figure 5b & 6b). Peaks I-III from BSA LKT also contained a prominent 66,000 band, consistent with BSA, which did not react with MAB to LKT on western blots (Figure 6b). It is likely that this band is composed primarily of BSA.

Unconcentrated fractions from Peaks II and III from BSA-LKT did not contain a readily detected 97,000 band on SDS-PAGE (Figure 6a), however a 97,000 protein was barely discernible on western blots (Figure 6b). The 97,000 protein band was visible in concentrated Peaks II and III on western blots (Figure 7) and SDS-PAGE. SDS-PAGE and western blots of peaks I, II and III for RPMI-LKT/BSA were similar to BSA-LKT (Figure 8a & 8b). Judging from the band densities, although Peak I contains much higher amounts of the 97,000 LKT subunit, it is much less active than Peaks II or III which contain lower amounts of the 97,000 LKT subunit. Based on K_{av} values, Peak I (regardless of the source) consisted of a large aggregated form of LKT with a molecular weight $\gg 669,000$. The K_{av} for Peaks II and III correspond to molecular weights $\geq 65,000$ (Peak II) and $< 29,000$ (Peak III) (Figure 9), values less than the LKT subunit weight (97,000), suggesting that K_{av} may not reflect separation only by molecular size.

The identity of fractions from Peaks II and III were further confirmed as LKT by target cell specificity and neutralization with a MAB to LKT. Peaks I, II and III from BSA-LKT and RPMI-LKT/BSA were cytolytic to bovine lymphoma cells but not to equine WBC's (Table 2a). Cytolytic activity in all three peaks was neutralized by a MAB to LKT (Table 2b), confirming that Peaks II and III as well as Peak I consist of LKT.

Figure 5. SDS-PAGE and Western Blot from RPMI-LKT. (A) Silver stained SDS-PAGE gel of ammonium sulfate precipitated (AS) LKT and Peak I from RPMI-LKT had a 97,000 protein as the prominent band. **(B)** Western blot of the duplicate SDS-PAGE gel, immunoblotted with MAB605b. The prominent band for Peak I and AS LKT was a 97,000 protein.

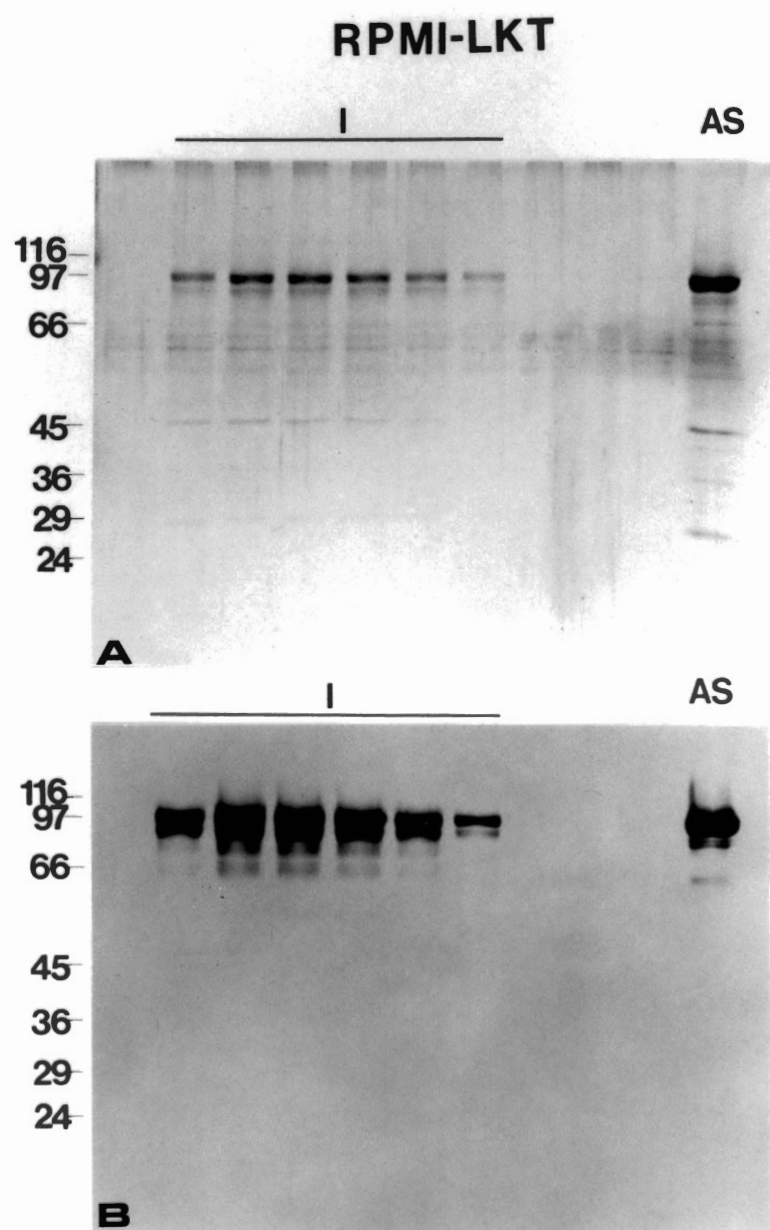


Figure 6. SDS-PAGE and Western blot of unconcentrated fractions from BSA-LKT. (A) SDS-PAGE: Peaks I, II and III and ammonium sulfate precipitated (AS) LKT from BSA-LKT contained multiple bands, with the prominent band in Peak I being a 97,000 protein; Peaks I-III also contained a prominent 66,000 protein. **(B) Western Blot:** the prominent band in Peak I was a 97,000 protein. In Peaks II and III the 97,000 protein was barely discernible, and the 66,000 band did not react with the MAB. The AS BSA-LKT band contained smeared protein bands between 97,000 and 43,000.

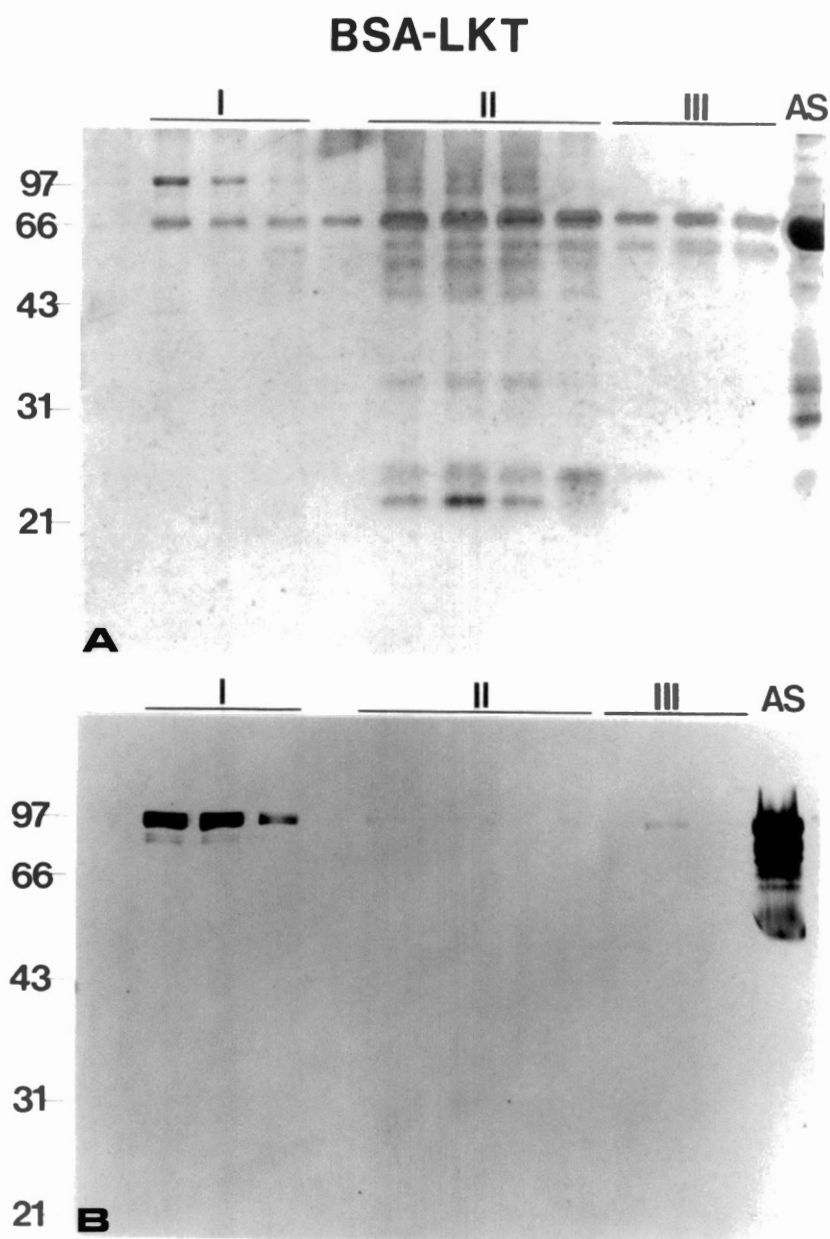


Figure 7. Western blot of pooled fractions from BSA-LKT, Peaks I, II and III, blotted with MAB601. **(A)** Pooled, non-concentrated fractions from Peaks I, II, and III. Peak I contained a prominent 97,000 protein. Peaks II and III had no visible bands. **(B)** Pooled, concentrated Peaks I, II and III. The prominent band in Peaks I-III was a 97,000 protein band. **(C)** Supernate from TCA precipitated, pooled fractions. Peak I had a prominent 97,000 protein. Peaks II and III contained no visible bands. **CS** Culture supernate from BSA-LKT had a prominent 97,000 band. **AS** Ammonium sulfate precipitated BSA-LKT had a smeared protein band between 43,000 and 97,000.

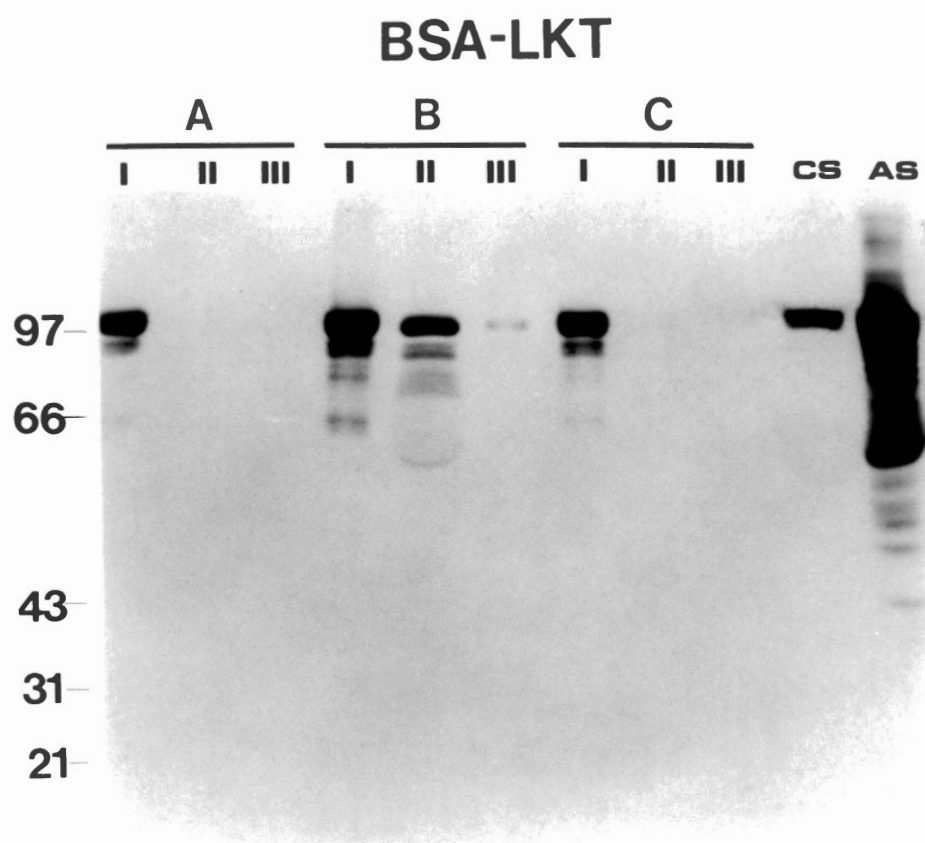


Figure 8. SDS-PAGE and Western Blot of RPMI-LKT/BSA. (A) SDS-PAGE: ammonium sulfate precipitated (AS) LKT and Peak I from RPMI-LKT/BSA had a prominent 97,000 band, and Peaks I, II and III had a 66,000 protein band. **(B) Western Blot:** a 97,000 protein band was present in Peak I; Peaks II and III had no visible bands.

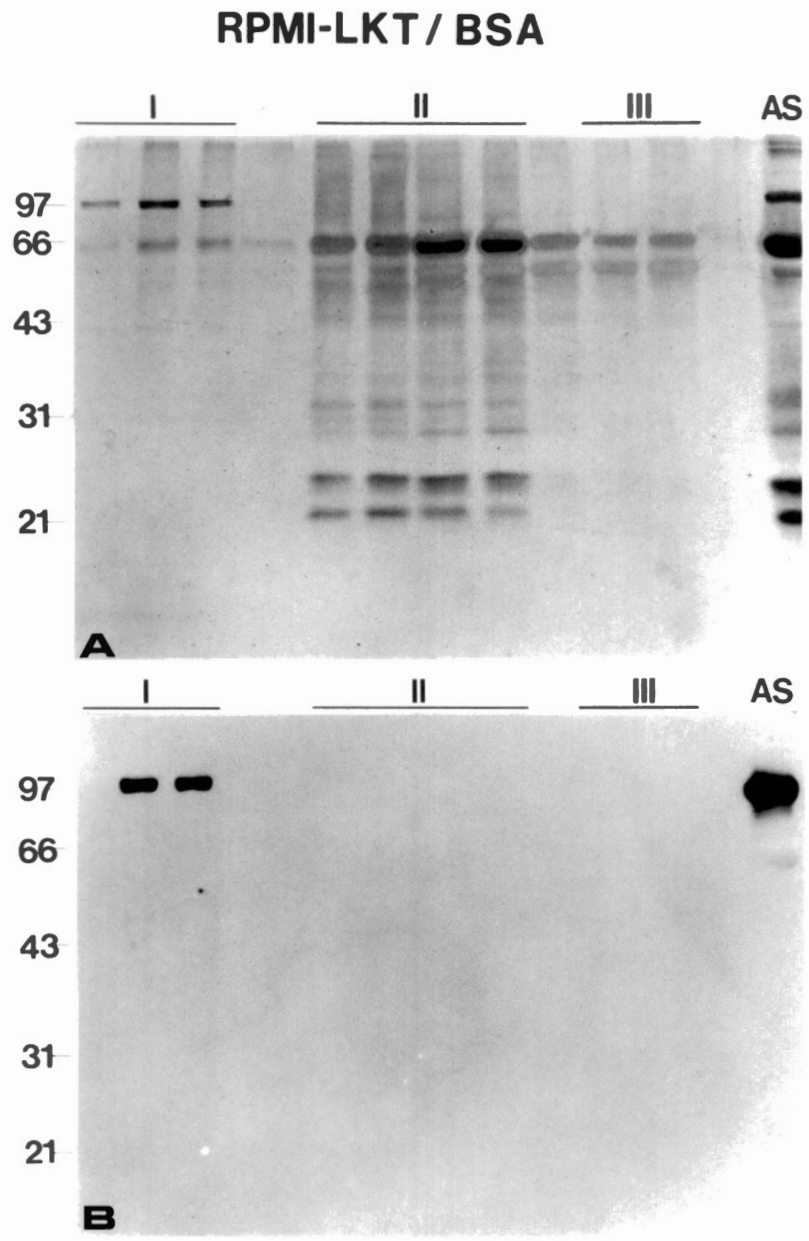


Figure 9. Calibration Curve for Sephacryl HR-400, illustrating the relationship between K_{av} and molecular weight for Peaks I, II and III. The K_{av} corresponded to a molecular weight $> 669,000$ for Peak I, $> 65,000$ for Peak II, and $< 29,000$ for Peak III.

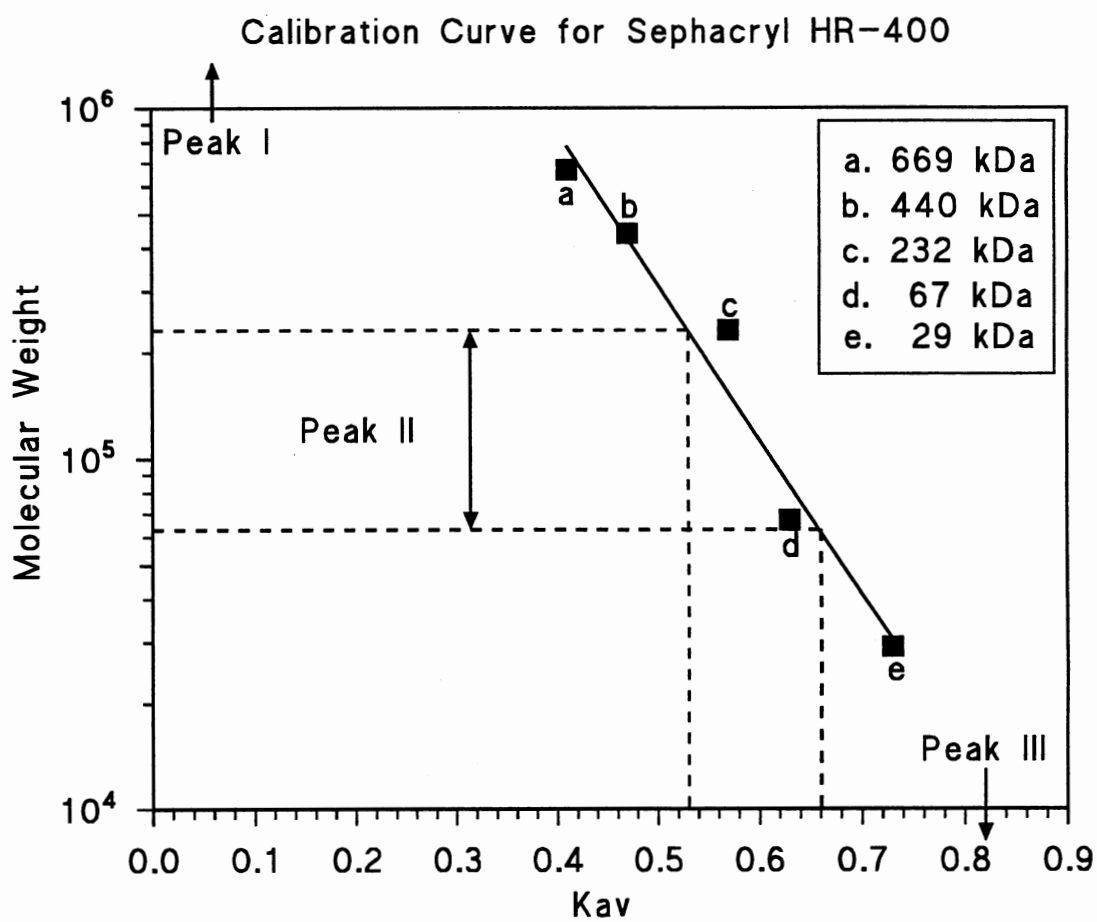


TABLE 2: Summary of (a) Target Cell Specificity Cytotoxicity Assays and (b) Neutralization Assays for Peaks I, II and III of Fractionated BSA-LKT

	PEAK I	PEAK II	PEAK III
(a) % Specific Release ^a (Equine WBC)	0.0 ± 0.0	0.0 ± 0.0	3.6 ± 1.5
Control % Specific Release ^a (BL-3 cells)	58.8 ± 8.1	49.9 ± 6.9	42.6 ± 6.8
(b) % Specific Release ^a (+MAB601)	0.0 ± 0.0	0.1 ± 0.14	0.8 ± 0.6
Control % Specific Release ^a (-MAB601)	65.6 ± 7.2	70.0 ± 4.6	42.6 ± 5.5

^a%Specific release (mean ± standard deviation) =

$$\frac{(\text{X LDH release BL-3 cells or Equine WBC} - \text{Spontaneous LDH release BL-3 cells or Equine WBC})}{(\text{Maximum LDH release BL-3 cells or Equine WBC} - \text{Spontaneous LDH release BL-3 cells or Equine WBC})} \times 100$$

All test values had $P < 0.05$ as compared with results with control BL-3 cells (a) or control BL-3 cells - MAB (b).

DISCUSSION

Native LKT produced by *P. haemolytica* cultures grown in RPMI medium appears to be a large aggregate form with low activity. Previous studies have attempted to purify native LKT and determine its molecular weight. Our findings are in agreement with previous studies which found that LKT is a large protein with a molecular weight $> 150,000$ (14,18) by gel filtration and $> 300,000$ molecular weight by ultrafiltration (2). Recently, the *E. coli* α -hemolysin, a genetically related toxin, has been shown to exist in the form of large aggregates, held together by hydrophobic forces, and that the large aggregate form is relatively inactive (19).

Disaggregation of the native large aggregated *E. coli* α -hemolysin increases its activity (19). Treatment of α -hemolysin with chaotropic agents, such as urea, increase the cytolytic activity and decreases the size of the aggregates, possibly by disruption of the hydrophobic forces which would otherwise force the toxin into the aggregated form (19). Our study indicates that a similar phenomenon may be occurring with the *P. haemolytica* LKT. BSA added directly to the RPMI-LKT may change the environment of the LKT such that the native molecule becomes more amphipathic and disaggregates, enhancing toxic activity and producing multiple molecular toxin forms on gel filtration. In addition to enhancing the activity of LKT, BSA probably enhances production of the LKT by *P. haemolytica* grown in RPMI-BSA. BSA-LKT had significantly higher LKT activity than either RPMI-LKT/BSA or RPMI-LKT. BSA may be causing other changes in the toxin as well. By changing the hydrophobicity of the native molecule, it may

enhance the native toxin's affinity for the column matrix, causing it to chromatograph on the gel filtration column, thus explaining why Peak III elutes at less than 29,000 on gel filtration, yet contains a 97,000 protein band on western blots.

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